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Immune Response to Vaccinia Virus Recombinants Expressing Glycoproteins gE, gB, gH, and gL of Varicella-Zoster Virus

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Immunogenicity of Varicella-zoster virus glycoproteins gE, gB, gH, and gL expressed by recombinant vaccinia viruses (VV) separately or simultaneously was determined in mice and guinea pigs by ELISA, Western blotting, radioimmunoprecipitation, plaque reduction assay, and skin test. Single VV-gE and VV-gB recombinants and double VV-gH/gL recombinant elicited specific antibodies with VZV neutralizing activity in mice. Co-expression of gE and gB by one recombinant VV resulted in an increased antibody response in comparison with immunization with single recombinants or their mixtures. Unlike anti-gB and anti-gH/gL antibodies, the gE-specific antibodies had no virus neutralizing activity in absence of complement, and when used alone, they even caused considerable increase of VZV infectious units. Moreover, immune sera containing anti-gE antibodies antagonized complement independent virus-neutralizing activity of anti-gB- and anti-gH/gL-positive sera. The ability to induce delayed hypersensitivity reaction to VZV antigens was observed after immunization of guinea pigs with gE- and/or gB-expressing VVs.

Key Words: varicella-zoster virus; herpesviruses; glycoproteins; vaccinia virus; vaccine; antibodies; cellular imunity; virus neutralization; immunization.

INTRODUCTION

Varicella-zoster virus (VZV) is responsible for chickenpox and shingles. The host reactivity to VZV involves both humoral and cell-mediated immune responses (for review, see Arvin, 1995). The major viral antigens of VZV are the glycoproteins (gps), gE, gB, gH, and gL, which are structural components of the virion envelope. Glycoprotein gE (M_r 90–98 kDa) is the most abundant viral gp in VZV-infected cells (Montalvo and Grose, 1985). This glycoprotein is highly modified by both N- and O-linked sugars, sialiation, myristylation, palmitylation and sulphation, as well as by serine/threonine and tyrosine phosphorylation (Grose, 1990; Harper and Kangro, 1990; Namazue *et al.,* 1989). In infected cells, VZV gE forms a noncovalently linked complex with VZV gI (Yao *et al.,* 1993). Owing to two targeting signals (Alconada *et al.,* 1996; Zhu *et al.,* 1996), gE is targeted to *trans*-Golgi network (TGN). The gE molecule binds Fc fragment of IgG. Usually, antibodies against gE are able to neutralize VZV infection *in vitro* in the presence of complement only (Grose and Litwin, 1988; Ludvíková *et al.,* 1991); however, complement-independent neutralizing monoclonal gE antibodies have been also prepared (Wu and Forghani, 1997). The second most strongly immunogenic glycoprotein of VZV, gB, is a heterodimer of two polypeptide chains (M_r 66 and 65 kDa) that are linked by disulphide bonds. Molecule of gB contains both N- and O-linked sugars, and it is sialiated, sulphated, and palmitylated (Grose, 1990). The gB was found to bind to cell-surface heparan sulphate proteoglycans (Jacquet *et al.,* 1998). Glycoprotein gB elicits complement-independent virus-neutralizing antibodies (Edson *et al.,* 1985a,b). The gB-specific antibodies inhibit VZV-induced cell-to-cell fusion in infected monolayers (Montalvo and Grose, 1987). The third most abundant VZV glycoprotein, gH, is associated with another viral glycoprotein, gL, with chaperone function (Forghani *et al.,* 1994), and this complex is transported to the cell surface (Duus *et al.,* 1995). The gH:gL complex is responsible for viral entry, egress, cell fusion, and cell-to-cell spread of the VZV in cultured cells. Glycoprotein gH induces complement-independent neutralizing antibodies. Two other glycoproteins, gC and gI, induce low-level immune responses (Huang *et al.,* 1992). Also some non-glycosylated viral proteins like IE62 or thymidine kinase are significant targets of host-immune responses. Although cell-mediated immune response is more important for limiting the spread of virus and clearing infection (Arvin, 1995), antibodies also substantially interfere with VZV replication *in vivo* as is evident from reduction of severity of varicella after administration of high-titer VZV immunoglobulin (Zaia *et al.,* 1983). The exact role of particular viral antigens in induction of immune status is not well understood. It has been shown that antibody responses to gE and gB after natural infection and after vaccination with OKA vaccine is highly variable from one individual to another; however, these

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TABLE 1

VV-VZV Recombinants Used in Immunization Experiments

Recombinant VV	Promoter	Insertion site in VV genome: TK gene	Insertion site in VV genome: HA gene
VV-gE	7.5	gE	—
VV-gB	11k	gB	—
VV-gE/gB	7.5/11k	gB	gE
VV-gH	7.5	gH	—
VV-gL	11k	gL	—
VV-gH/gL	7.5/11k	gH	gL

two glycoproteins are predominant among other viral antigens in respect to induction of VZV-neutralizing antibodies (Haumont *et al.,* 1997).

We have recently shown that VZV glycoproteins gE and gH:gL expressed by recombinant vaccinia virus (Ludvíková *et al.,* 1991; Němečková *et al.,* 1996) elicit antibodies with virus-neutralizing activity in mice. The goals adressed in this paper were to better characterize the main immunogennic VZV glycoproteins that are supposed to be included into a subunit vaccine against VZV, to determine the contribution of the particular glycoproteins to humoral and cellular immune responses, and to evaluate the influence of coexpression of the glycoproteins. For this purpose, we prepared a set of single and double VV recombinants expressing four VZV genes (gE, gB, gH, and gL) and used them for immunization of mice and guinea pigs. Humoral and cell-mediated immune responses to VZV antigens were determined.

RESULTS

Antibody response of mice inoculated with gE- and gB-expressing recombinant VVs

To evaluate the immunogenicity of VV-VZV recombinants, the antibody responses of mice inoculated with various doses of different recombinants (Table 1) and of their mixtures were analyzed. Results of the examination of sera of mice after administration of VV-expressing gE and gB are shown in the upper part of Table 2. The results obtained by ELISA and WB indicate that both glycoproteins are relatively good immunogens in mice since the administration of 1.10^6 PFU (Groups 1 and 2) of either VV-gE or VV-gB induced specific antibodies. As observed for the VV-gB recombinant, the amount of antibodies formed depended on the dose of the recombinant virus administered but only within a certain range of doses (Groups 2–4). The data on gE and gB antibodies monitored by ELISA and WB were in good agreement. To test the immunogenicity, mixtures of the two recombinants containing one dose of VV-gE (1.10^6 PFU) and increasing doses of VV-gB (1.10^6, 3.10^6, or 10.10^6 PFU) were co-injected (Groups 5–7). To further determine the

TABLE 2

Antibody Response of Mice Inoculated with Recombinant VVs Expressing gE and gB

Group No.	Dose of virus inoculated i.p. into mice	$ELISA_{[Abs.\ 492\ nm]}$[a] Antibodies to: VZV antigen[b]	Antibodies to: gE[c]	Antibodies to: gB[d]	$WB_{[OD]}$ Antibodies to: gE[e]	Antibodies to: gB[e]	Neutralization titer of sera: Complement −	Complement +
1	VV-gE 1.10^6[f]	1.00 ± 0.19[g]	0.90	0.09	114	0	<4[h]	8
2	VV-gB 1.10^6	0.54 ± 0.14	0.07	0.56	0	9	4	8
3	VV-gB 3.10^6	0.65 ± 0.21	0.06	0.69	0	38	128	512
4	VV-gB 10.10^6	0.60 ± 0.32	0.21	0.72	0	48	128	256
5	VV-gB 1.10^6 + VV-gE 1.10^6	1.17 ± 0.25	0.83	0.60	75	4	<4	16
6	VV-gB 3.10^6 + VV-gE 1.10^6	1.30 ± 0.19	0.71	0.78	32	11	16	128
7	VV-gB 10.10^6 + VV-gE 1.10^6	0.96 ± 0.28	0.54	0.74	11	11	8	256
8	VV-gE/gB 1.10^6	1.42 ± 0.33	1.09	0.73	130	21	4	512
9	VV-gB 1.10^6 + VV-gL 1.10^6	0.50 ± 0.08	—	—	0	13	8	32
10	VV-gB 3.10^6 + VV-gL 1.10^6	0.75 ± 0.13	—	—	0	44	128	512
11	VV-gB 10.10^6 + VV-gL 1.10^6	0.72 ± 0.25	—	—	0	62	128	512
12	VV-gL 10.10^6	0.06 ± 0.13	—	—	0	0	<4	<4

[a] Antigen prepared from VZV-infected cells was used in all ELISA tests.
[b] Non-absorbed individual mouse sera.
[c] Pooled sera were absorbed with antigen prepared from VV-gB-infected CV-1 cells.
[d] Pooled sera were absorbed with antigen prepared from VV-gE-infected CV-1 cells.
[e] Intensity of staining of gE 98-kDa band and gB 120-kDa band was determined densitometrically.
[f] Amount of recombinant virus administered (PFU).
[g] Standard deviation.
[h] Reciprocal of highest serum dilution that neutralized ≥50% of VZV plaques.

TABLE 3

Antibody Response of Mice Immunized with VVs Expressing gH and/or gL as Detected by RIPA

Group No.	Dose of virus inoculated i.p. into mice	Peak area of gp precipitated [OD]		
		gH (118 kDa)	gL (19 kDa)	pre-gH (97 kDa)
1	VV-gL 1.10^7	0	0	0
2	VV-gH 1.10^7	0	0	0
3	VV-gH 1.10^7 + VV-gL 1.10^7	0	0	0
4	VV-gH/gL 1.10^7	8204	1893	2450
5	VV-gH/gL 1.10^6	7549	1426	2494
8	VV-gH/gL 1.10^6 + VV-gE/gB 1.10^6	4612	1151	1735
9	VV-gH/gL 1.10^6 + VV-gE/gB 3.10^6	2627	530	897
10	VV-gH/gL 1.10^6 + VV-gE/gB 10.10^6	1514	0	390

effect of co-administered virus, VV-gE was replaced by the control VV-gL virus (Groups 9–11). VV-gL was chosen because it does not induce VZV-specific antibodies reactive in the tests used (Group 12). As evidenced by both ELISA and WB, the presence of increasing amount of the second VV in the mixture reduced the antibody response to the gE antigen. Inoculation of a mixture of VV-gE and VV-gB recombinants and in particular the double VV-gE/gB recombinant induced higher levels of antibodies against the complex VZV antigen than did any single VV recombinant tested. The VZV-neutralizing activity of sera from all groups immunized with gE- and/or gB-expressing VVs was measured using plaque reduction test. In agreement with previous reports, sera of mice immunized with VV-gE neutralized VZV only in the presence of complement (Group 1). On the other hand, gB-specific virus-neutralizing antibodies were detected both in the presence and absence of complement, and the magnitude of neutralization titers was in good agreement with the values found by the other tests used (Groups 2–4). The highest virus-neutralizing activity was found in sera of mice immunized with the double VV-gE/gB recombinant (Group 8) or high doses of the VV-gB recombinant in the presence of complement (Groups 3, 10, and 11). Surprisingly, administration of VV-gE in mixtures with various amounts of VV-gB, or immunization with recombinant VV-gE/gB, resulted in decreased levels of antibodies capable of neutralizing VZV in the absence of complement (Groups 5–8), although the gB-specific antibody levels present in such sera were rather high (e.g. in Group 8). A low complement-independent virus-neutralizing capacity of sera was not observed if VV-gL was used instead of VV-gE in the mixture (Groups 9–11).

Antibody response in mice inoculated with gH- and/or gL-expressing recombinant VVs

Antibodies against gH were determined using the radioimmunoprecipitation assay since they could not be detected by either ELISA or WB. Bands corresponding to glycoproteins gH gL and pre-gH were measured densitometrically, and OD values of their peak areas are shown in Table 3. We did not observe any specific band precipitated by sera of mice inoculated with the VV-gL (Group 1) or VV-gH (Group 2) single recombinants, given alone or together (Group 3), at a dose of 1.10^7. As for the VV-gH/gL double recombinant, a dose of 1.10^7 PFU (Group 4) induced slightly higher levels of anti-gH and anti-gL antibodies than did a dose of 1.10^6 (Group 5). The sera precipitated both a fully processed 118-kDa form of gH combined with a 19-kDa form of gL and a premature 97-kDa form of gH. If the mice were immunized with mixtures of both double recombinants VV-gH/gL and VV-gE/gB, we observed that increasing the dose of latter virus in the mixture resulted in decrease of the antibodies elicited by the former virus (Groups 8–10). This is an observation similar to that seen after immunization with the mixtures of VV-gE and gB (Table 2).

The virus-neutralizing capacities of sera of mice inoculated with the single VV-gL or VV-gH recombinant, with their mixtures, or with the double recombinant VV-gH/gL were also compared. Administration of single recombinants or their mixtures at a dose of 1.10^7 PFU did not elicit virus neutralizing antibodies of a titer ≥ 4 (not shown). The presence of high titers of virus-neutralizing antibodies after immunization with 1.10^6 PFU of double recombinant VV-gH/gL (Table 4, Group 4) confirmed that the genes coding for gH and gL must be expressed simultaneously in the same cell to process the gH:gL immunogen properly. We compared the efficacy of the administration of both double recombinants VV-gE/gB and VV-gH/gL mixed at various ratios (Table 4). The simultaneous inoculation of both viruses (Groups 1–3) did not result in an increase of virus neutralizing antibody titers in comparison with the inoculation of one recombinant only (Groups 4–7). Raising the VV-gE/gB virus dose in mixtures (Groups 2 and 3) resulted in a reduction of complement-independent virus-neutralizing antibodies. A similar negative effect of anti-gE antibodies was seen also in Experiment 1 shown in Table 2 (Groups 5–7). This decrease of titers either could be the result of

TABLE 4

In Vitro Neutralization of VZV with Sera of Mice Immunized with the Double Recombinant VVs Expressing gE/gB and/or gH/gL

Group No.	Dose of virus inoculated i.p. into mice	Neutralization titer of sera (Complement) −	+
1	VV-gH/gL 1.10^{6a} + VV-gE/gB 1.10^6	128[b]	128
2	VV-gH/gL 1.10^6 + VV-gE/gB 3.10^6	64	128
3	VV-gH/gL 1.10^6 + VV-gE/gB 10.10^6	16	256
4	VV-gH/gL 1.10^6	128	128
5	VV-gE/gB 1.10^6	<4	128
6	VV-gE/gB 3.10^6	8	256
7	VV-gE/gB 10.10^6	16	256

[a,b] For explanations, see Table 2.

a negative influence of unequal doses of the two viruses in the mixtures as recently reported (Kutinová *et al.,* 1999) or could be due to some specific influence of anti gE antibodies on the virus. To elucidate this effect, we tested the influence of anti-gE-positive sera in the absence of complement in more detail.

Enhancement of VZV infection *in vitro* by anti-gE-positive sera in the absence of complement

When anti-VV-gE sera were titrated in the absence of complement, we repeatedly observed an increase in the VZV plaque count in comparison with untreated virus. This effect was exhibited by the same serum dilutions which were active in VZV neutralization in the presence of complement (Fig. 1A). Relative plaque numbers were expressed as percentages of plaque counts in serum-free controls. To see whether gE-specific antibodies could influence sera containing gB- or gH/gL-specific antibodies, we tested the effect of various serum mixtures. The effect of anti-gE antibody presence on the activity of complement-independent anti-gB antibodies is shown in Fig. 1B. Both sera and their mixture neutralized VZV in the presence of complement. Addition of anti-gE antibodies to various dilutions of anti-gB serum subsequently reduced the virus-neutralizing activity of anti-gB antibodies in the absence of complement. The continuous increase of the plaque number was associated with the decreasing concentration of anti-B antibody in mixtures. Similarly, in the absence of complement anti-gE, immune sera inhibited the neutralization activity of both concentrations (1:16, 1:32) of VV-gH/gL serum used, as is shown in Fig. 1C. This enhancement was dependent on the amount of anti-gH/gL-neutralizing antibody. The effect was anti-gE specific, and it was not observed for any other sera of mice immunized with recombinants such as VV-gB, VV-gH, VV-gL, VV-preS2-S or nonrecombinant VV TK^-.

Cutaneous reaction to VZV antigens

Cellular immune responses of guinea pigs immunized with recombinant VV were tested by the skin test. Cutaneous reactions were measured 48 h after intradermal inoculation of test antigen. The results of two immunization experiments are shown in Table 5. In the first experiment, guinea pigs were infected with seven different recombinant VVs, a mixture of two double recombinants, extracellular VZV-Oka, or with PBS. The table shows mean cutaneous reaction values ± standard deviation (SD). As compared with reactions after VV-preS2S inoculation taken as a background negative control, weakly positive cutaneous reactions were induced in animals immunized with VV-gE or VV-gB. The differences from negative control were not statistically significant from what could be caused by great variability of animal responses. However, we observed several times that positive reactions to VZV antigen were accompanied by more intensive colouring of erythema (not shown). In animals immunized with VV-gE/gB or with a mixture of VV-gE/gB + VV-gH/gL, the mean values of skin reaction size differed from the control group significantly ($P = 0.029$ and $P = 0.009$, respectively). The response in animals immunized with VZV-Oka also showed a significant difference ($P = 0.021$). No positive cutaneous reaction was observed in controls injected with PBS only or in animals infected with the single recombinants VV-gH, VV-gL or, surprisingly, with the double recombinant VV-gH/gL.

Sera of all animals were tested for the presence of anti-VZV and anti-VV antibodies 5 weeks after immunization. Inoculation of recombinant VVs elicited anti-VV responses in all animals. All sera also contained VZV-neutralizing antibodies. Since the sera of groups inoculated with PBS and VV-preS2S displayed VZV-neutralizing activity at titers of 8–16, it is obvious that at least a portion of these antibodies was produced in response to the administration of skin-test antigens. A finding of relatively high neutralization activity in sera from animals immunized with VV-gE/gB, VV-gE, or VV-gB confirmed an immunizing capability of VV-expressed gE and gB in guinea pigs. Unlike mice, guinea pigs were apparently less capable of responding to VV-expressed gH/gL by production of VZV-neutralizing antibodies, and this might have been associated with their failure to develop skin reactivity. To investigate this point further and to confirm the most important data from Experiment I, another experiment (Table 5, Experiment II) was carried out in which VVgE/gB and VVgH/gL recombinants were used for immunization and the skin test was only performed in a portion of the animals. We found no cutaneous reaction and no antibodies reacting with VZV in neutralization test in VV-gH/gL-inoculated animals, which were not boosted by skin test antigen.

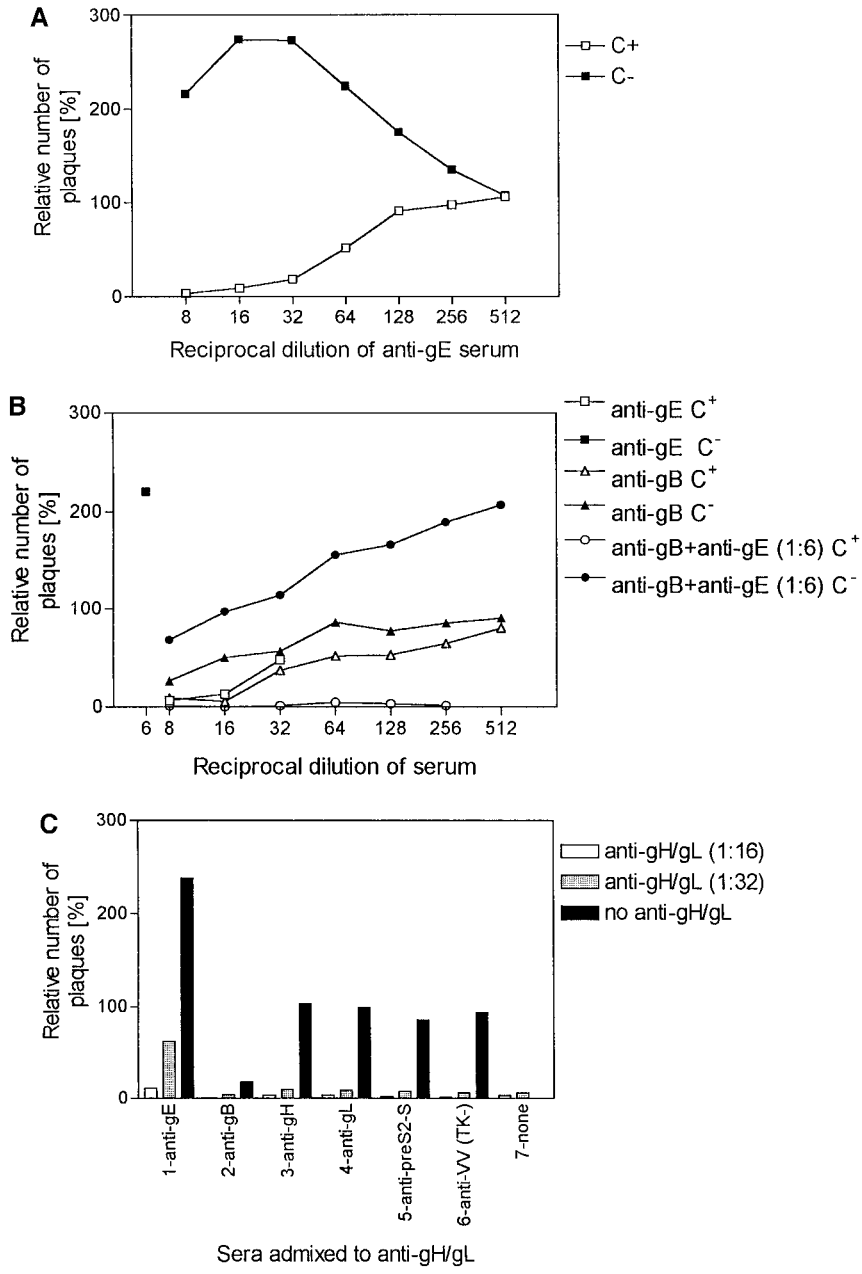

FIG. 1. Enhancement of VZV plaque formation by immune sera of mice inoculated with VV-gE. (A) Plaque reduction and plaque enhancement by gE-positive sera in the presence (-□-) and absence (-■-) of complement, respectively. The gE-positive sera were pooled from mice immunized with 1.10^6 VV with the gE gene inserted into hemagglutinin of VV. The relative numbers of plaques in all three graphs represent percentages of plaque counts in samples without serum. (B) Inhibition of complement-independent virus neutralization activity of anti-gB-positive serum by anti-gE positive serum. One dilution (1:6) of anti-gE-positive serum (after immunization with VV-gE) was mixed with serial dilutions (1:8–1:512) of anti-gB-positive serum. Virus neutralization activity was tested either in the presence (C^+) or absence (C^-) of complement. (C) Inhibition of complement-independent virus neutralization activity of anti-gH/gL-positive serum by anti-gE-positive serum but not by other sera. Sera of mice immunized with 1-VV-gE, 2-VV-gB, 3-VV-gH, 4-VV-gL, 5-VV-preS2-S, or 6-VV(TK^-) diluted 1:6 were mixed with serum of mice immunized with VV-gH/gL, diluted, 1:16 or 1:32, or the sera were used unmixed (no gH/gL). The last two bars (7-none) represent virus neutralization by unmixed anti-gH/gL. The mixed sera and controls were assayed by the plaque reduction test in the absence of complement.

DISCUSSION

Immune response to particular glycoproteins following infection with VZV has been well documented (Weigle and Grose, 1984; Brunell *et al.,* 1987; Dubey *et al.,* 1988; Arvin *et al.,* 1991; Haumont *et al.,* 1997), but data on immunogenicity of separately expressed VZV glycoproteins are less numerous (Vafai and Yang, 1991; Vafai, 1993; Ludvíková *et al.,* 1991; Lowry *et al.,* 1992; Massaer *et al.,* 1993; Němečková *et al.,* 1996). The results of the present series of experiments demonstrated that VV recombinants expressing VZV gE, gB, or gH + gL glyco-

TABLE 5

Cutaneous Reaction and Antibody Responses in Guinea Pigs Immunized with VV-VZV Recombinants

Virus	N^a	Skin reaction		Antibodies$_{[Abs\ 492\ nm \pm SD]}$		Neutralization titer of sera C^+
		Area [mm^2]	P	VZVag	VVag	
			Experiment I[b]			
VV-preS2S	3	20.0 ± 24.9	—	0.196 ± 0.117	0.434 ± 0.305	8
VV-gE/gB	3	57.7 ± 8.5	0.029[c]	0.613 ± 0.141	0.151 ± 0.103	128
VV-gH/gL	3	−9.3 ± 19.0	0.110	0.300 ± 0.061	0.176 ± 0.100	16
VV-gE/gB + VV-gH/gL	4	76.5 ± 17.0	0.009*	0.592 ± 0.082	0.209 ± 0.098	128
VV-gE	3	100.7 ± 73.5	0.083	0.514 ± 0.128	0.291 ± 0.100	64
VV-gB	3	107.0 ± 112.9	0.183	0.393 ± 0.087	0.156 ± 0.098	32
VV-gH	3	−21.0 ± 36.3	0.113	0.253 ± 0.106	0.296 ± 0.065	16
VV-gL	3	48.3 ± 16.5	0.107	0.250 ± 0.120	0.423 ± 0.182	16
VZV-Oka	3	176.3 ± 98.0	0.021*	0.336 ± 0.070	0.026 ± 0.015	128
PBS	4	0 ± 0		0.296 ± 0.031	0.028 ± 0.017	16
			Experiment II[d]			
VV-gE/gB	3	72.5 ± 7.7	0.002*	0.588 ± 0.129	0.135 ± 0.063	128
VV-gE/gB[e]	3	n.d.	—	0.259 ± 0.039	0.151 ± 0.053	16
VV-gH/gL	4	8.0 ± 8.0	—	0.326 ± 0.100	0.236 ± 0.205	16
VV-gH/gL[e]	3	n.d.	—	0.006 ± 0.010	0.226 ± 0.170	<4

Note. Area values are means ± SD.

[a] Number of animals in group.

[b] In Experiment I, 60 μg of VZV antigen was intradermally injected 18 days after administration of recombinant VV. Skin reaction area was measured 48 h after injection of antigen. Guinea pigs were bled out 5 weeks after inoculation of recombinant VV. VZV- and VV-specific antibodies were determined in individual sera. VZV-neutralizing activity was measured in pooled sera. Cutaneous reactions in animals inoculated with the various VV-VZV recombinants were compared with animals immunized with the VV-preS2S recombinant.

[c] Statistical significance was tested by the unpaired *t* test. * Indicates that the size of cutaneous reaction areas differed significantly from animals originally inoculated with VV-preS2S (in the 95% confidence interval).

[d] The animals in Experiment II were injected on the same time schedule and with the same doses of VV and VZV antigen as in Experiment I. Significance of the differences between cutanous reactions in VV-gE/gB- and VV-gH/gL-injected animals was tested.

[e] Skin test was not performed.

protein genes were able to induce gE-, gB-, and gH-specific antibodies, respectively, which displayed VZV-neutralizing activity. Immunization with single recombinants or with mixtures of single recombinants was, in all combinations, less efficient than immunization with double recombinants. It had already been known for some time that to induce anti-gH neutralizing antibodies, gH had to be expressed together with gL in the same cell; doses lower than 2.10^7 PFU of VV-gH and VV-gL administered intraperitoneally did not induce virus-neutralizing antibodies in mice (Němečková *et al.,* 1996). In the present experiments, immunization with the VV-gE/gB double recombinant resulted in much higher titers of neutralization antibodies than those obtained with mixtures of the corresponding single recombinants. This might have been a consequence of temporary interaction between these two glycoproteins in the course of their synthesis; it should be remembered that a complex, heterooligomeric organization of glycoproteins has also been found in other herpes viruses (Handler *et al.,* 1996). However, our efforts to co-precipitate the gE/gB complex 24 h after infection with VV-gE/gB and crosslink it with dithio-*bis*(succinimidylpropionate) (Marešová, results not shown) have not been successful so far. Immunization with mixtures of two double recombinants, VV-gE/gB and VV-gH/gL, did not increase neutralization titers in postinfection sera. Cellular immune responses to glycoproteins gE, gB, and gH have been found in subjects with acute and convalescent varicella (Arvin *et al.,* 1986; Giller *et al.,* 1989) as well as in guinea pigs inoculated with the Oka varicella vaccine (Sato *et al.,* 1998). We observed that glycoproteins gE and gB expressed by VV were able to induce delayed-type hypersensitivity in guinea pigs similarly as did immunization with extracellular VZV-OKA. We could not find any significant skin reaction after immunization with VV-gH/gL or VV-gH plus VV-gL, although the presence of VV-specific antibodies in all guinea pigs indicated that all animals were infected with VV. Furthermore, the titers of anti-VV antibodies in animals inoculated with VV-gH/gL, VV-gB, or VV-gE/gB did not differ markedly. It could be speculated that the amount of gH/gL formed was sufficient to stimulate virus-neutralizing antibodies in mice but failed to elicit neutralizing antibodies and delayed-type skin reaction in guinea

pigs. It is likewise possible that the processing of these two glycoproteins is less efficient in guinea pig than in murine cells.

We also observed that when anti-gE-positive sera were used in the absence of complement in neutralization assays, the number of plaques increased. Antibody-dependent enhancement (ADE) of virus infection *in vitro* was described for several viruses, including human immunodeficiency virus type 1 (Homsy *et al.,* 1990), influenza virus (Ochiai *et al.,* 1990), and respiratory syncytial virus (Gimenez *et al.,* 1989). ADE in these viruses can be explained so that the exposed Fc portions of the antibodies that are bound to virus particles bind to Fc receptors on the surfaces of cells, thereby increasing the internalization of the virus. This explains the mechanism of ADE in macrophages or monocytes but is not likely for VZV infection of fibroblastoid LEP cells used in our study. We also established that ADE was not due to an enhanced stability of the gE-antibody-treated virus (results not shown).

At this writing, the nature of this observation is not clear; however, it might be associated with some other phenomena described recently. It has been observed by Shiraki *et al.* (1997) that postinfection treatment of cells with the gE:gI complex isolated by affinity chromatography inhibited VZV plaque formation. The effect was dose dependent and treatment of VZV-infected cells with 100 μg of purified glycoprotein reduced plaque number by 85%. VZV glycoproteins (Shiraki and Takahashi, 1982; Montalvo and Grose, 1986), including gE (Marešová *et al.,* 2000), have been shown to be released from cells infected with VZV or recombinant VV-gE, and therefore the cell-free virus stocks prepared by sonication of VZV infected cells may contain such inhibitory gE:gI complexes. It follows that the actual titer of virus in different stocks may depend on the concentration of infectious virions and at the same time on the concentration of inhibiting gE:gI complexes. Thus, the role of anti-gE antibodies in ADE could be dependent on the binding of the inhibitory antigens present in the preparations of cell-free VZV used in the plaque reduction test. Formation of antigen–antibody complexes could prevent the inhibitory effect of free gE:gI on VZV infection and cause an increase in the plaque count *in vitro.* The differences in the content of free VZV glycoproteins in cell-free virus stocks used in different laboratories for neutralization tests might be responsible for different findings concerning the capability of anti-gE antibody to neutralize VZV without complement. The other mechanism operative may involve the nature of gE antibodies used. Polyclonal antibodies elicited by VV-gE recombinants may have a higher affinity to soluble gE:gI complexes than monoclonal antibodies used by other investigators.

Another possible mechanism of VZV infection enhancement by anti-gE could be based on the fact that gE is internalized from the membrane of infected cells by endocytosis (Olson and Grose, 1997). It has been observed that antibodies to gE of pseudorabies virus, another alphaherpes virus, induced endocytosis and caused bridging and capping of membrane glycoproteins on infected cells (Favoreel *et al.,* 1999). It is possible that increased ligand-induced endocytosis could result in a higher rate of infection.

Our results suggested that all three glycoproteins, gE, gB, and gH/gL, would be important components for a future subunit VZV vaccine. Although neither gE nor gB required co-synthesis of other glycoproteins so essentially as gH required gL, the positive immunization effect of VV-gE/gB suggested that interaction of viral glycoproteins could occur during their synthesis or processing and that the simultaneous synthesis of recombinant glycoproteins in the same cell could be worth considering in designing a subunit vaccine.

MATERIALS AND METHODS

Viruses and cells

All recombinant vaccinia viruses (VV) were prepared from clone P13 generated from smallpox Sevac VARIE vaccine (strain Praha) (Kutinová *et al.,* 1995). A list of single and double recombinants containing VZV gene 37 (gH), gene 60 (gL), gene 31 (gB), or gene 68 (gE) inserted in the VV thymidine kinase (TK) or hemagglutinin (HA) genes is shown in Table 1. Expression of extrinsic genes was controlled by VV early/late p7.5k or late 11k promoters. Construction of single recombinant VVs expressing VZV gE, designated VV-gE (Ludvíková *et al.,* 1991) and expressing gH and gL and designated VV-gH and VV-gL, respectively (Němečková *et al.,* 1996), were described previously. Single recombinant VV-expressing gB and double recombinants VV-gE/gB and VV-gH/gL were prepared recently (Marešová *et al.,* 2000). Electrophoretic pattern of glycoproteins produced by recombinants used in the present study have been shown in the recent paper by Marešová *et al.* (2000). Glycoproteins gE, gB, and gH seemed to be produced in similar amounts. The VV-preS2-S-expressing middle envelope protein of Hepatitis B virus was prepared from plasmid pM3 as described previously (Kutinová *et al.,* 1994). Thymidine-kinase-deficient VV denoted VV TK^- is a spontaneous mutant of P13 virus. Human-embryo diploid cells (LEP) and monkey-kidney cells (CV-1) were used for the growth of recombinant viruses. All cells were cultivated in modified medium 199 (EPL) containing bovine serum growth-active proteins but no complete serum (Michl, 1961). The VVs used for immunization experiments were grown in the chorioallantoic membranes of 11-day-old chicken embryos and partially purified by the modified method of Joklik (1962), (Kutinová *et al.,* 1999). VZV (strain Zuzana) was isolated in our laboratory from skin vesicle of a child with varicella. VZV-Oka was kindly provided by Dr. A. Sauerbrei, Erfurt. Both VZV strains were propagated in LEP cells.

Immunization of mice

Four-week-old outbred female mice, strain CD-1 (ICR)BR (Charles River), were injected intraperitoneally with 0.5 ml PBS containing recombinant VV. Ten mice were immunized in each group. Five weeks later the mice were anesthetized with halothane (Narcotane, Léčiva, Praha) and bled out. Sera used in neutralization test were inactivated at 56°C for 30 min.

Testing of antibodies

ELISA. Levels of VZV- and VV-specific antibodies in individual mouse sera were determined by ELISA as described previously (Ludvíková *et al.,* 1991). For the detection of specific gE and gB antibodies in mice immunized concurrently with these two VZV glycoproteins, pooled sera diluted 1:10 were absorbed prior to testing. The antigens for absorption were prepared from CV-1 cells infected with either VV-gB or VV-gE, and the absorption was performed as described previously (Němečková *et al.,* 1996).

Western blotting. Specific gE and gB antibodies were quantified by Western blotting. In brief, LEP cell cultures infected with VZV were harvested 2 days after infection when the CPE had reached 90%. The cells, washed twice with PBS, were scraped into PBS, centrifuged at 3000 *g* and lysed in Laemmli buffer with 2-mercaptoethanol. Samples were incubated at 95°C for 5 min and applied on top of a 7.5% PAAG using a wide single-well comb. The proteins separated were transferred to a nitrocellulose membrane using the semidry electrophoretic method. The membrane was stained with Ponceau S and cut into 5-mm strips. The strips, preincubated with 10% non-fat dry milk in PBS for 1 h, were incubated with mixtures of the sera being tested, diluted 1:50, at 4°C overnight. After washing (PBS, 0.2% Tween 20), the strips were incubated with swine anti-IgG labeled with horseradish peroxidase, washed, and stained with diaminobenzidine. The maxima of the 98-kDa band and the 120-kDa band corresponding to gE and the uncleaved gB molecule, respectively, were scanned and quantified using the Scan PacK 3.0 programme (Biometra).

Radioimmunoprecipitation. Antibodies to gH and gL proteins were determined using immunoprecipitation of [^{35}S]methionine- and cysteine-labeled VZV antigens prepared by the procedure described previously (Němečková *et al.,* 1996). The extracts were prepared from monolayers of CV-1 cells infected with VV-gH/gL or VV-gH recombinant or parental VV. Mixed sera from 10 mice, diluted 1:6, were absorbed with wt VV-infected cells before testing as already described (Němečková *et al.,* 1996). Monoclonal antibody V3 (Sugano *et al.,* 1991), obtained through the courtesy of T. Sugano, Tokyo, was used to detect processed gH in complex with gL and a zoster-patient serum was used to recognize premature gH. Autoradiograms were scanned and density of specific bands (118 and 97 kDa for gH and 19 kDa for gL) was quantified using the Scan PacK 3.0 programme (Biometra).

Neutralization assay

The VZV-neutralizing capacity of sera was examined by a plaque reduction test. The cell-free virus used in the assay was prepared similarly as was described by Ilobi and Martin (1989). In brief, VZV (strain Zuzana)-infected LEP cells exhibiting about 50–70% c.p.e. were scraped into E-MEM supplemented with 5% sucrose, 0.1% sodium glutamate, and 10% thermoinactivated newborn calf serum. The resulting cell suspension was sonicated (Soniprep, MSE) at amplitude 14 μm for 30 s and centrifuged at 950 *g* for 15 min. The supernatant fluid was used as cell-free virus preparation, and it was stored in aliquots at −65°C. In the neutralization test, the viruses were diluted 1:20–1:30 to achieve 100 ± 50 PFU in control cultures. Several cell-free virus stocks were used in the present study, but the data in each table or figure were always obtained with the same cell-free virus preparation. Equal volumes of virus, serial dilutions of heat-inactivated mouse serum and 1:4 diluted guinea pig serum, as source of complement, were mixed and incubated for 1 h at 34°C. To test complement-independent antibodies, the heat-inactivated (56°C/30 min) guinea pig serum was used in the mixtures. In the control mixtures, the serum tested was replaced by medium. A volume of 0.3 ml of each mixture was inoculated on two 60-mm Petri dishes with LEP cell monolayer at room temperature and 30 min later 5 ml of culture medium (E-MEM, 10% heat-inactivated newborn calf serum) was added. After 10 days of incubation at 37°C, the medium was removed and cells were stained (E-MEM, 1% calf serum, 0.05% neutral red) at room temperature for ≥1 h. Plaques were counted immediately after removal of staining medium. The neutralizing titer was expressed as the reciprocal of the highest serum dilution causing ≥50% plaque number reduction in relation to control.

Skin test in guinea pigs

Groups of three to four outbred female guinea pigs (BFA strain) weighing 225–250 g were injected with two separate doses of 5.10^6 PFU of recombinant VV in 0.5 ml PBS given simultaneously intraperitoneally and subcutaneously in the back. Control animals received PBS only. One group of animals was injected subcutaneously with 6.10^3 PFU of extracellular VZV-Oka. Skin tests were performed 18 days after immunization. Skin test antigens were prepared according to the modified method of Kamiya *et al.* (1977). In brief, the OKA strain of VZV was used for preparation of skin-test antigen. Monolayers of LEP cells with freshly changed medium EPL were inoculated with virus-infected cells at a ratio of one infected cell to four uninfected cells. After incubation at 37°C for

48 h, the cells were washed three times with PBS and fed with serum-free DMEM medium. The cultures were incubated at 37°C for 5 h and again washed three times with PBS. Cells were scraped off, suspended in PBS, and the suspension was centrifuged at 600 *g* for 10 min. The sediment was resuspended in PBS, sonicated in a Soniprep MSE (amplitude 12 μm) for 2 min and centrifuged at 3000 *g* for 20 min. The supernatant was UV-irradiated for 10 min to inactivate infectious virus. Control antigen was prepared from noninfected cells by the same procedure. The materials were kept at −65°C until used. Volumes of 50 μl of positive or control skin test antigens containing 60 μg of total protein were injected intradermally in either flank of locally shaved animals. Cutaneous reaction was measured 48 h after injection, and its size for each animal was calculated using following formula: Area = (long diameter$_{VZV}$ × short diameter$_{VZV}$) − (long diameter$_{control}$ × short diameter$_{control}$). The data were statistically analysed using unpaired *t* test.

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